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Increased Levels of Tumor Necrosis Factor α Are Associated with an Increased Risk of Cytomegalovirus Infection after Allogeneic Hematopoietic Stem Cell Transplantation

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ABSTRACT

Tumor necrosis factor-α (TNF) has been implicated in the reactivation of cytomegalovirus (CMV) at a cellular level. We therefore hypothesized that increased posttransplantation TNF levels may be associated with the development of CMV antigenemia (CMV-Ag). We studied 134 patients undergoing allogeneic hematopoietic stem cell transplantation. After excluding CMV-negative donor and recipient pairs, 94 patients were evaluable. By cluster analysis, 2 groups were designated by TNF levels obtained between days 4 and 7 after transplantation: 58 patients had low levels (median, 0 pg/mL; range, 0-5.5 pg/mL), and 36 patients had high levels (median, 43.75 pg/mL; range, 7.5-1756 pg/mL). To determine the independent effect of TNF on the development of CMV-Ag and acute graft-versus-host disease and on survival, Kaplan-Meier and Cox models stratified by TNF patient groups were evaluated. High TNF levels were associated with a more rapid onset of CMV-Ag ($P < .001$) and with the occurrence of the composite end point of CMV-Ag or death ($P < .001$). Factors independently associated with CMV-Ag in multivariate analysis were a high TNF level (hazard ratio [HR], 2.57; $P = .003$) and acute graft-versus-host disease (as a time-dependent covariate; HR, 2.30; $P = .010$). Factors independently associated with the composite end point of CNV-Ag or death were a high TNF level (HR, 2.42; $P < .001$) and patient age (per year; HR, 1.93; $P = .017$). In conclusion, a high posttransplantation TNF level is significantly associated with the risk for developing CMV infection. Early detection of high levels of TNF may be used to identify patients at high risk for developing CMV-Ag.

KEY WORDS

Cytomegalovirus • Hematopoietic stem cell/bone marrow transplantation • Tumor necrosis factor α

INTRODUCTION

Hematopoietic stem cell transplantation (HSCT) is an important therapeutic modality for malignant and nonmalignant disorders. Patients are immunocompromised after transplantation because of conditioning, immunosuppressive drugs, or graft-versus-host disease (GVHD) [1]. A major complication of transplantation immunosuppression is cytomegalovirus (CMV) infection, which contributes significantly to morbidity and mortality after transplantation [2-4]. Several groups have demonstrated that CMV disease may be reduced by improved surveillance and preemptive antiviral therapies; however, CMV infection continues to be a major concern after transplantation [5-9]. The deleterious effects of CMV infection include indirect effects of CMV reactivation that are due to immunosuppressive status induced by CMV; these lead to a risk of other infections [10]. In addition, drugs such as foscarnet and ganciclovir to treat CMV reactivation have toxic effects, including renal failure or neutropenia, that predispose to invasive bacterial or fungal infections [11,12].

Tumor necrosis factor-α (TNF) has an important role in the pathogenesis of a variety of inflammatory disorders [13]. Studies have indicated a role for TNF in major transplant-related complications, including veno-occlusive disease, pneumonitis, and severe endothelial leakage syndrome [14]. In HSCT, both murine

models and clinical studies suggest that TNF released after conditioning is involved in the pathogenesis of acute GVHD (aGVHD) [14-19]. Furthermore, use of transplanted T cells from a TNF-deficient donor abrogates the development of idiopathic pneumonia syndrome, thus establishing the requirement for donor-derived TNF in a murine model [20]. Collectively, these studies suggest that TNF is a significant mediator in the pathogenesis of posttransplantation complications. However, the association between TNF and the subsequent development of CMV infection is not known.

After allogeneic stem cell transplantation (SCT), patients are at risk for CMV infection because of a variety of factors, including donor and/or recipient CMV serologic status and immunosuppressive medications [21]. The occurrence of aGVHD with associated use of corticosteroids increases the risk of developing CMV infection [21]. Because TNF has been shown to stimulate CMV reactivation at a cellular level in a dose-dependent manner [22,23], we hypothesized that high TNF levels in the immediate posttransplantation period may be associated with subsequent development of CMV infection, independently of GVHD. With the recent advent of anti-TNF therapies [24-26], finding an association between TNF and CMV infection may be clinically relevant to identify patients at high risk for developing CMV infection and initiate a preemptive strategy of antiviral treatment.

MATERIALS AND METHODS

Patients

We prospectively collected blood samples and clinical data from 134 patients who underwent allogeneic HSCT. For the purpose of this study, we excluded 32 CMV-negative donor and recipient (D−/R−) pairs (of which only 1 patient developed CMV-Ag). An additional 8 patients were excluded because they had missing TNF levels in the first week after transplantation. The demographics of the patients are described in Table 1. Most patients (68%) received peripheral blood stem cells, and the remainder (32%) received bone marrow or cord blood transplants. Sixty-three patients had transplants from related and 31 from unrelated donors. Patients were treated with myeloablative (65%) or nonmyeloablative (35%) conditioning regimens based on institutional protocols or treatment plans. GVHD prophylaxis consisted of tacrolimus or cyclosporin A along with minidose methotrexate. Patients who developed aGVHD grades II to IV were treated with methylprednisolone 2 mg/kg with a scheduled taper of 25% every 4 days, depending on response. If no response was noted, the dose was held and/or additional immunosuppressive agents such as daclizumab, mycophenolate mofetil, and/or investigational agents were indicated. The primary study end point was the appearance of CMV antigenemia (CMV-Ag). Secondary study end points included patient death, the composite end point of patient death or CMV-Ag, aGVHD (grades II-IV), and CMV disease. Patients were followed up until death, the last follow-up date, or the study end point of June 18, 2004. The median follow-up was 1103 days (range, 70-1792 days).

Table 1. *Patient Characteristics*

Variable	Data
n	94*
Recipient male	60.6%
Donor male	55.3%
Patient age, y (mean ± SD)	46.5 ± 12.3
Donor age, y (mean ± SD)	44.6 ± 13.0
Recipient positive	77.7%
Donor positive	68.1%
Myeloablative conditioning	64.9%
Related donor transplant	67.0%
Peripheral blood stem cell graft source	68.1%
Survival rate (within 150 and after transplantation)†	
Patient survival	61.5%
CMV-Ag	45.6%
Acute GVHD (within 100 d)	34.8%

*Excluding CMV pretransplantation D−/R− pairs from 134 patients total.

†Kaplan-Meier survival rate at 150 days after transplantation.

TNF Measurements

Blood samples were collected twice during the first week after transplantation between 4 and 7 days. Plasma samples were stored frozen at −200°C until analysis. Plasma TNF levels were measured by enzyme-linked immunosorbent assay according to standard protocols and reagents (Endogen, Woburn, MA). All cytokine assays were performed in duplicate or triplicate (according to sample availability), and the average is reported in picograms per milliliter. On the basis of the average of the sample results obtained between days 4 and 7, the patients were grouped into low- or high-TNF cluster groups, as described in the statistical methods.

CMV Surveillance and Preemptive Therapy

CMV-Ag testing was performed through day +100 by using the pp65 antigenemia assay that detects the said antigen by immunofluorescence [11]. After day 100, CMV-Ag was monitored in patients considered to be at high risk on the basis of active GVHD and in those taking immunosuppressive medications. Positive CMV-Ag was treated with ganciclovir 5 mg/kg intravenously twice daily for 2 weeks followed by 5 mg/kg intravenously daily for 1 week. Alternatively, valganciclovir 900 mg orally twice daily for 2

weeks was used, followed by 900 mg daily for 1 week. If antigenemia persisted after 4 weeks of treatment or if it increased after 3 weeks, treatment was switched to foscarnet.

Clinical Study End Points

The primary study end point was CMV infection, defined as positive CMV-Ag within the first 150 days. Secondary end points were clinical CMV disease and the composite outcome of CMV-Ag and death. Criteria for CMV disease have been previously defined as pneumonia, gastrointestinal disease, or end-organ localization [5,27]. Acute GVHD grade II to IV occurring before day 100 after transplantation was defined according to consensus criteria [28]. Conditioning regimens were defined as ablative if high-dose therapy was administered. High-dose therapy is that after which marrow recovery would not be expected without treatment. All other regimens with reduced intensity were defined as nonmyeloablative.

Statistical Methods

We classified TNF-level categories by using cluster analysis on the log-transformed counts {log [TNF (pg/mL) + 1]}. We chose to divide the patients according to high and low TNF levels. To designate the cutoff level for high versus low TNF levels, a univariate disjoint clustering method based on euclidean squared distances was used for the transformed variables. The clustering algorithm minimized the absolute difference between the data and the corresponding cluster medians. Similar statistical procedures were conducted for classifying the CD3 and CD34 cells infused. The 2 TNF categories were analyzed with univariate Kaplan-Meier models and Cox proportional hazard models, adjusted for relevant covariates, to assess their association with outcomes of interest. The log-rank test was used to test the association of TNF group levels and the time to the applicable outcome. Models were constructed for the outcomes of CMV-Ag onset, patient death, composite CMV-Ag, patient death, and CMV disease onset. Survival models for CMV onset were truncated at 150 days because there were no CMV-Ag events after this point and to maintain the proportionality of covariate factors as in the Cox model assumptions. We used a stepwise backward variable-elimination method similar to that of Wahlby et al. [29] to construct our multivariate model. The *P* value threshold of remaining in the model was .15. Covariates entered into the models were sex match, patient age (treated as a continuous variable), disease risk level (high or standard), graft source (bone marrow or peripheral blood stem cell), related or unrelated donor, number of CD34 cells infused, number of CD3 cells infused, donor (D) and recipient (R) pretransplantation CMV combination (D+/R−, D+/R+, and D−/R+), conditioning type, and days to onset of aGVHD as a time-dependent covariate. We also examined the association of CMV-Ag and TNF levels in several strata that were significantly disproportionately represented in the 2 TNF groups. To further ascertain the viability of the group delineation by the cluster technique, we produced a receiver operating characteristic (ROC) curve plot of the transformed TNF levels. All analyses were conducted with SAS software (version 9.0; SAS Institute, Cary, NC).

RESULTS

TNF Levels and Patient Characteristics

Two TNF categories were determined by cluster analysis with ranges of 0 to 5.5 pg/mL for the low group (n = 58) and 7.5 to 1756 pg/mL for the high group (n = 36). The clustered distribution of TNF levels is shown in Figure 1. The characteristics of patients in the entire group and in the 2 groups of TNF are shown in Tables 1 and 2, respectively.

CMV Infection

CMV-Ag occurred in 44 (46.8%) patients, all within 150 days after transplantation. The incidence of positive CMV-Ag was significantly lower in the D+/R− CMV transplants: only 2 (9.5%) of 21 patients of this type contracted CMV-Ag (log-rank *P* value comparing the 3 groups = .0019). The incidence rates in the D+/R+ (25/43; 58.1%) and D−/R+ (17/30; 56.7%) groups were similar (log-rank *P* = .8097). Twenty-two (38%) patients in the low-TNF group developed CMV-Ag, and 22 (61%) patients in the high group developed CMV-Ag within the first 150 days after transplantation. By Kaplan-Meier analysis, the time to CMV-Ag onset was significantly associated with TNF levels (*P* = .0008; Figure 2). High TNF levels were associated with a higher

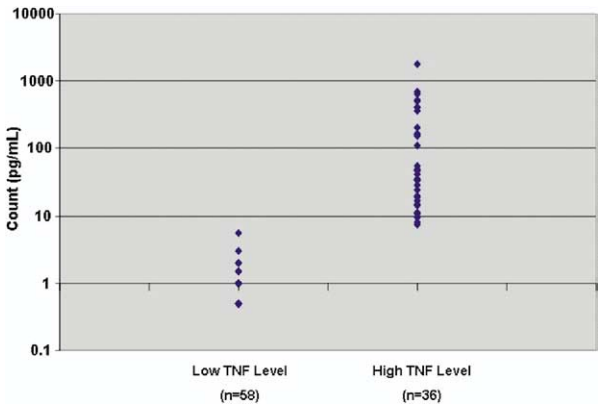

Figure 1. Patient groups by cluster analysis of posttransplantation TNF levels. Zero values are not shown on the log chart.

Table 2. *Characteristics of Patient Groups by TNF Levels*

Demographic Characteristic	Low-TNF Group (n = 58)	High-TNF Group (n = 36)	*P* Value
Patients			
Sex match (%)	63.8	52.8	.290
Recipient age, y (mean ± SD)	46.1 ± 12.2	47.0 ± 12.7	.740
High disease risk (%)*	91.4	91.7	.961
Transplant type			
Myeloablative conditioning (%)	65.5	63.9	.872
Peripheral blood stem cells (%)	58.6	83.3	.013
Related donor transplant (%)	50.0	94.4	<.001
CMV pretransplantation status			
D^+ (%)	63.8	75.0	.257
R^+ (%)	75.9	80.6	.595
Graft characteristics			
High CD3 count (%)†	43.1	63.9	.050
High CD34 count (%)‡	74.1	88.9	.083
No GVHD onset (%)	46.6	41.7	.6068§
Grade I GVHD (%)	3.5	11.1	
Grade II GVHD (%)	34.5	27.8	
Grade III GVHD (%)	13.8	16.7	
Grade IV GVHD (%)	1.7	2.8	

*High risk: acute myelogenous leukemia (AML) and acute lymphoblastic leukemia (ALL) other than first complete remission (CR1), primary induction failure; acute leukemia from antecedent hematologic disorder; chronic myelogenous leukemia not in first chronic phase (CML not CP1); chronic lymphocytic leukemia; acute bilineage leukemia; non-Hodgkin lymphoma; Hodgkin disease; myelodysplastic syndrome (MDS) not refractory anemia (RA) or ringed sideroblasts (RARS); multiple myeloma; eosinophilic leukemia (n = 1). Low risk: ALL CR1; AML CR1; CML CP1; MDS RA, RARS.

†Median CD3 count: low-TNF group, 5×10^8/kg; high TNF, 5.1×10^8/kg.

‡Median CD34 count: low TNF, 1.8×10^6/kg; high TNF, 2.6×10^6/kg.

§Chi-square test for the association of GVHD grade and TNF group.

incidence of CMV-Ag in related-donor transplantations (log-rank $P = .0245$), peripheral blood graft source transplantations (log-rank $P = .0018$), and high CD3 count recipients (log-rank $P = .0006$).

By Cox regression, high TNF levels were independently associated with CMV-Ag (hazard ratio, 2.57; 95% confidence interval [CI], 1.37-4.82) when adjusting for possible confounding variables (Table 3). Acute GVHD was a significant time-dependent covariate with an adjusted hazard for CMV-AG conversion of 2.3 (95% CI, 1.22-4.33). Relative to transplantations in which both the donor and recipient were positive for CMV, transplantations with donor positive and recipient negative were protective for CMV-Ag onset (adjusted hazard ratio [AHR], 0.11; 95% CI, 0.03-0.47).

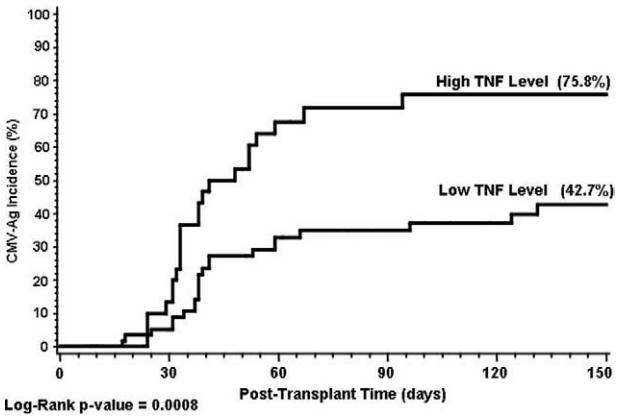

Figure 2. Kaplan-Meier plot of development of CMV-Ag and correlation with TNF levels.

Composite End Point

By Kaplan-Meier analysis, the time to CMV-Ag onset or death was significantly associated with TNF levels ($P = .0001$; Figure 3). By Cox regression, the high TNF level was significantly associated with the composite end point of CMV-Ag or death with an AHR of 2.42 (95% CI, 1.48-3.97). Also significant in the model were aGVHD onset (as a time-dependent covariate; AHR, 1.93; 95% CI, 1.13-3.31) and D+/R− CMV pretransplantation status (AHR, 0.39; 95% CI, 0.19-0.80). Progression of disease was the single most common cause of death in both groups.

CMV Disease

CMV disease occurred in 5 (8.6%) patients in the low-TNF group and 4 (11.1%) in the high-TNF group, but this was not statistically significant in Kaplan-Meier analysis ($P = .2441$). There was no significant association between donor/recipient pretransplantation CMV combination and TNF group (χ^2 $P = .3134$).

Predictive Value of TNF Levels for the Composite End Point of CMV-Ag Onset or Death

We estimated the utility of using first-week TNF cytokine levels as a predictive diagnostic criterion for

Table 3. *Multivariate Cox Model for Outcome of Time to CMV-Ag Onset*

Variable (Reference Group)	Level	Hazard Estimate	95% CI	*P* value
TNF level (low)	**High**	**2.57**	**1.37-4.82**	**.003**
Acute GVHD onset	**Time-dependent**	**2.30**	**1.22-4.33**	**.010**
Donor/recipient CMV pretransplantation status (D+/R+)*	**D+/R−**	**0.11**	**0.03-0.47**	**.003**
	D−/R+	**0.96**	**0.51-1.81**	**.894**
Patient age	**Per year**	**1.02**	**1.00-1.05**	**.095**
Sex match (no)	**Yes**	**0.61**	**0.33-1.13**	**.116**

CI indicates confidence interval.

*Excluding pretransplantation CMV D−/R− pairs.

CMV-Ag and the composite end point of CMV-Ag or death in the 100 days after transplantation. For this analysis, patients had to have a minimum of 100 days of posttransplantation follow-up (n = 91). Therefore, if a patient died before day 100, the patient was still included until the day of death and subsequently removed from the analysis. Of the 36 patients in the high-TNF group, 22 (61%) developed CMV-Ag, as opposed to 20 (36.4%) of 55 in the low-TNF group (χ^2 P = .0206). Thirty patients (83%) in the high group experienced CMV-Ag or death within the first 100 days after transplantation, whereas 30 (54.6%) experienced the combined end point in the low group (χ^2 P = .0046). To further illustrate the relationship between the first-week TNF count and the outcomes of CMV-Ag or patient death, we produced an ROC plot with the sensitivity and 1 − specificity of particular TNF levels. The area under the ROC plot was 0.622 (Figure 4), representing the overall predictive value or proportion of concordant observations between TNF levels and subsequent outcomes in the sample.

DISCUSSION

TNF has been demonstrated to stimulate CMV reactivation at a cellular level in a dose-dependent manner, but the clinical significance of this finding has been uncertain. Our study suggests that increased levels of TNF in the early posttransplantation period are associated with a significant risk for the development of CMV infection that is independent of the onset of aGVHD. Murine and human studies have suggested that early TNF release due to conditioning-related tissue damage is associated with posttransplantation complications, particularly aGVHD [16,30]. Patients with aGVHD have an increased risk of CMV infection; however, a direct link between CMV and TNF in SCT recipients was unknown. Increased plasma TNF levels and CMV-Ag have been documented in solid-organ transplant recipients [23,31,32]. In renal transplant recipients, upregulation of CD4 T-cell membrane-bound and soluble TNF responses was seen with CMV disease [33]. In addition, an association of increased systemic TNF levels and CMV reactivation has been found in nontransplant conditions such as sepsis, cirrhosis, and autoimmune disorders [22,31,34,35]. However, our study is the first to describe the risk association between TNF and CMV infection in SCT recipients. In fact, our study demonstrates that 76% of patients with high early TNF levels develop CMV-Ag within the first 100 days after transplantation (Figure 2). Even more striking was the association of increased TNF with the composite end point of CMV-Ag or death; 92% of patients with high TNF reached this end point within 150 days (Figure 3). The multivariate Cox model confirmed that this association was independent of other risk factors for CMV and was notably independent of the onset of aGVHD, thus indicating that there is a direct link between TNF levels and the risk for CMV disease and death.

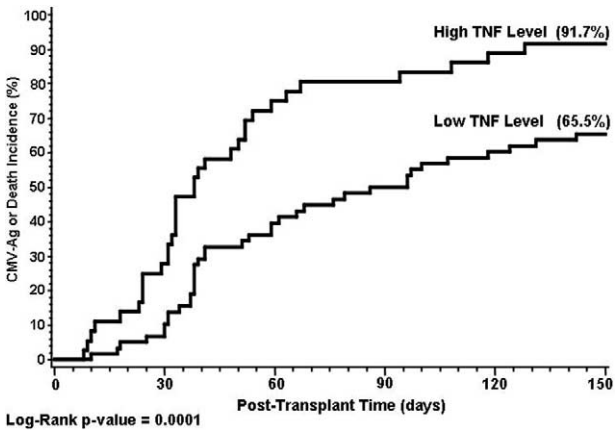

Figure 3. Kaplan-Meier plot for outcome of CMV-Ag onset or patient death by TNF group level (excluding CMV D−R− pairs).

CMV is a β-herpesvirus that can be reactivated from latency in immunocompromised states, such as in posttransplantation recipients or patients with acquired immunodeficiency syndromes. Understanding the immune mechanisms of CMV reactivation may lead to newer therapies against CMV infections. The mechanisms by which TNF and CMV interact in SCT recipients require further investigation. TNF has been shown to stimulate CMV reactivation at a cellular level in a dose-dependent manner [22,23]. Possible mechanisms for this association may be found

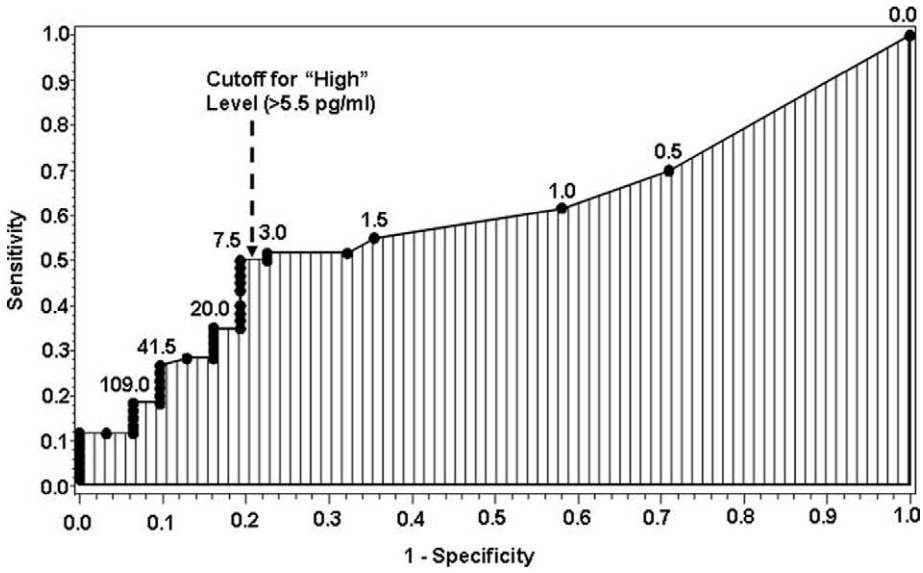

Figure 4. ROC plot of first-week TNF levels for the composite outcome of CMV-Ag or death within 100 days after transplantation. Values on the ROC plot indicate original TNF levels in picograms per milliliter.

from CMV pneumonitis animal models in transplantation, in which human CMV infection induces TNF release from alveolar macrophages through a natural killer cell and interferon γ–mediated immunopathology [36-38]. The upregulation of CMV infection by TNF is mediated via the TNF receptor 1 (p55) with a downstream signaling process that involves activation of protein kinase C and nuclear factor-κB (NFκB). The activated p65/p50 NFκB translocates into the nucleus and binds to the 18–base pair repetitive sequence motifs with the human CMV immediate early 1 enhancer region [31,39]. On the basis of these mechanisms, the role of TNF leading to CMV reactivation in our patients could be explained by the kidney transplant CMV model, in which induction of immediate early 1 gene expression is the first step in the reactivation of CMV [40]. This occurs as a result of an allogeneic response that induces the expression of TNF and subsequent activation of NFκB and activating protein 1 transcription factors [40]. This model and evidence of an association between CMV and myeloid cellular differentiation [41-43] suggest that the natural stimulus for CMV reactivation is an inflammatory response and that allogeneic transplantation mimics this inflammatory state.

Lack of CMV-specific cell-mediated immunity, such as lymphocyte function, is another important risk factor for CMV reactivation [5,44]. Monocytes are an important latent reservoir and may be susceptible to the TNF effect. Soderberg-Naucler et al. [45] have demonstrated that TNF and interferon γ specifically induce differentiation of monocytes into CMV-permissive macrophages resistant to the antiviral effects of these cytokines. However, in vitro studies by the same group supported a significant CMV-permissive role for interferon γ in monocytes rather than TNF [46]. Initial trials with blockade of TNF by using infliximab have yet to demonstrate a decrease in the incidence of CMV infection [24-26]. However, these patients are at great risk for opportunistic infections because of their immunosuppressed status from other agents, including corticosteroids [24-26]. These patients also have severe GVHD, and GVHD is a known risk factor for CMV infection. Therefore, reactivation of CMV is likely a multifactorial complex phenomenon in which TNF plays an important role but is not always tightly coupled.

Our study demonstrates that measurement of TNF as early as the first week after transplantation could be used as a clinical test to predict the development of CMV infection. Prior studies have suggested that cytokine levels may be intermittently increased during CMV infection [47,48]. A key difference between the Humar et al. [47] study and ours is that their study could not demonstrate an independent association between TNF and subsequent CMV infection. Their results demonstrate that TNF is increased around the time of development of CMV infection. This may be due to target organ damage during CMV infection. Conversely, our study found an association of early TNF levels with subsequent CMV activation. Our study does not prove a pathogenic link between TNF levels and CMV, but it legitimizes the hypothesis for such a link. Furthermore, earlier studies have evaluated TNF levels at a later time point—several days to weeks after engraftment. By this time point, many patients are at risk for developing GVHD and require corticosteroids or additional immunosuppression, which could alter TNF levels. Therefore, an advantage of our study is the measurement of TNF as part

of an early cytokine storm within days of transplantation, before the development of GVHD and additional immunosuppression and, more importantly, before the onset of CMV.

In our analysis, we excluded patients with a low risk of developing CMV infection, ie, CMV-seronegative recipients of CMV-seronegative donors. The reason for studying CMV-positive donors or patients and excluding CMV−/− was to study the patient population at risk for the ultimate outcome. As confirmed by our data, CMV−/− patients are at very low risk for CMV reactivation. For that reason, when looking for a clinically predictive test for CMV reactivation, the CMV-positive group is the appropriate study group.

In our multivariate analysis, there was a significant association between aGVHD and CMV-Ag (with aGVHD as a time-dependent covariate). Acute GVHD has been a documented risk factor for CMV-Ag in previous studies [49,50]. Our study suggests that TNF is independently associated with CMV-Ag, because we corrected for aGVHD as a time-dependent covariate. Not only was TNF associated with the onset of CMV-Ag, but it also performed well as a predictive test. In fact, clinically it could be quite useful to have a test within 7 days of transplantation to be able to predict CMV activation. Risk-stratifying patients before the development of CMV-Ag could reduce the direct and indirect toxicities of CMV infection and antiviral therapies. In fact, CMV prophylaxis instead of preemptive therapy might be justified in patients with early increases of TNF levels.

In summary, our results indicate for the first time that high posttransplantation TNF levels are an independent risk factor for the development of CMV infection independently of the onset of aGVHD. Because of the high predictive value of increased TNF levels as early as 1 week after transplantation for CMV infection and death, this could be a useful marker in identifying high-risk patients and targeting preemptive and preventative strategies.

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